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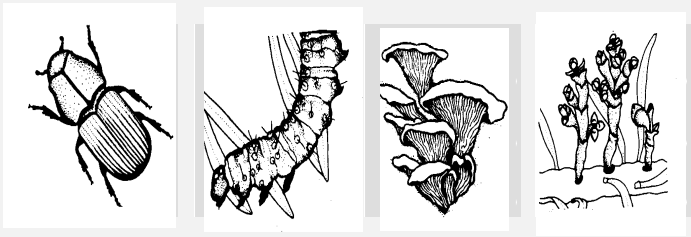

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PONDEROSA PINE SEED FUNGAL CONTAMINATION: EFFECTS OF STRATIFICATION AND STERILIZING TREATMENTS

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ABSTRACT

Eight ponderosa pine seedlots from the USDA Forest Service Nursery in Coeur d'Alene, Idaho were evaluated for mycoflora residing externally on their seedcoats. Seeds were selected from bulk storage and contained lots with large numbers of seeds planned for sowing at the nursery over the next few years. Two of the lots (7293 and 7295) were extensively contaminated with species of *Fusarium*, primarily *F. proliferatum*; other lots had relatively low *Fusarium* levels (0-3.5%). Other common seedcoat contaminating fungi included species of *Penicillium, Botrytis,* and *Trichoderma*. Representative seeds from both seedlots with extensive *Fusarium* contamination were sorted on the basis of size (small, medium, large), either stratified or unstratified, and either rinsed in tap water or treated with an aqueous bleach treatment. Bleach treatment greatly reduced contamination by *Fusarium* and *Penicillium* spp. on both seedlots. Higher levels of *Fusarium* and *Penicillium* were also recovered from stratified as compared to unstratified seed. Seed size did not affect level of fungal contamination. Larger seeds germinated at higher levels than either medium or small seeds. Bleach treatment did not, but stratification appreciably increased seed germination. Performance of ponderosa pine seedlots may be adversely affected by seedcoat contamination with *Fusarium* spp.

INTRODUCTION

Ponderosa pine (*Pinus ponderosa* Laws.) is an important forest tree species of the inland Northwest. Pine seedlings are produced at the USDA Forest Service Nursery in Coeur d'Alene, Idaho for reforestation on national forest lands in the Northern Region. One major limiting factor in seedling production is disease caused by several groups of fungi. An important disease affecting seed germination and seedling establishment is damping-off, which can occur either before seedling emergence (pre-emergence) or after seedlings have emerged above the groundline (post-emergence). One of the most important groups of fungi responsible for damping-off diseases is *Fusarium,* and several species are commonly responsible for damping-off at the nursery (James and others 1989). Although *Fusarium* spp. can be introduced into bareroot or container operations on soil or growing media (James 1984a; James and others 1990b), many potentially pathogenic

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isolates often contaminate outer seedcoats of seed and initiate disease either before or shortly after germination (James 1983, 1984b, 1989; James and Genz 1982; James and others 1996).

Growers sometimes encounter poor performing seedlots at the nursery. Seeds from such lots may have reduced germinability and resulting seedlings may exhibit higher than normal disease symptoms. Such seedlots may have high levels of potentially pathogenic fungi contaminating seeds, contributing significantly to disease problems (James 1983, 1989, 1995; James and Genz 1981, 1982).

Several production ponderosa pine seedlots recently exhibited higher than normal fungal contamination, evidenced by excessive mold growth in germination tests and poor performance in the nursery. Therefore, an evaluation was conducted to determine extent of seed-contaminating fungi and their potential role in germination of selected seedlots.

MATERIALS AND METHODS

Seeds from eight ponderosa pine seedlots were randomly collected from cold storage for evaluation of fungal contamination on external seedcoats. From 152-200 seeds were sampled per seedlot. Seeds were aseptically placed directly on the surface of an agar medium selective for *Fusarium* spp. and closely related fungi (Komada 1975). Seeds on agar media were incubated for 7-10 days under diurnal cycles of cool, fluorescent light at about 24°C, after which selected fungi emerging from seedcoats were transferred to potato dextrose and carnation leaf agar (Fisher and others 1982) for identification. The taxonomic scheme of Nelson and others (1983) was used to identify associated *Fusarium* spp.

Two of the seedlots with the highest levels of *Fusarium* contamination (7293 and 7295) were further evaluated. The effects of seed size, bleach, and cold stratification on seedcoat contaminants and subsequent seed germination were evaluated under controlled conditions. Another group of randomly selected seeds from bulk storage were visually segregated into three size classes: small, medium, and large. Seeds from each size class were either stratified for 28 days under cool, moist conditions, or left unstratified. No pre-stratification treatments were conducted. Subsamples of stratified or unstratified seeds were subjected to a standard 48-hour running-water rinse or soaked in an aqueous bleach solution for 10 minutes prior to rinsing (Wenny and Dumroese 1987). Bleach solutions consisted of one part commercial bleach mixed with two parts water. One hundred seeds were evaluated for each of the size/stratification/ water or bleach treatments. After treatments, seeds were aseptically placed on Komada's medium (20 per plate) and incubated as described above. Percentage seed colonization by *Fusarium* and other seedcoat-contaminating fungi was calculated after 10 days' incubation. During the fungal contamination assay, number of germinated seeds on the agar was noted (10-day germination). Standard nursery germination tests were also conducted on seeds following sizing and treatments. Two hundred seeds from each seedlot/size/stratification group/water or bleach treatment were incubated in germination chambers (dirurnal cycles of fluorescent light at about 21°C) for 21 days. Germination was monitored at 7, 14, and 21 days. Size and treatment effects on *Fusarium* and *Penicillium* seedcoat contamination and 10- and 21-day germination were analyzed with a one-way analysis of variance. Significant differences were separated using Duncan's multiple-range comparison test. All percentages underwent arc-sin conversion prior to analyses.

RESULTS AND DISCUSSION

Two of the eight ponderosa pine seedlots evaluated were severely infected with *Fusarium* spp. (table 1). Both these lots, 7293 and 7295, had about 35% and 29% of their seeds, respectively, colonized with these potentially

pathogenic fungi. The other six seedlots had low, more typical levels of *Fusarium* contamination. Experience has shown that some conifer seed may be infected with *Fusasrium* spp., but usually at levels well below 10% of assayed seed (Anderson and others 1984; Gabrielson 1988; James 1984b; James and Genz 1982). If more than 10% of sampled seeds are infected with *Fusarium*, treating seeds with surface disinfectants is usually recommended (Campbell and Landis 1990; James and Genz 1981).

The major *Fusarium* species colonizing seeds from all tested seedlots was *F. proliferatum* (Matsushima) Nirenberg, which comprised 97% of all the *Fusarium* spp. isolated. Other isolated fusaria included *F. sambucinum* Fuckel (1.5%), *F. acuminatum* Ell. & Ev. (0.75%), and *F. oxysporum* Schlecht. (0.75%).

For seedlots 7293 and 7295, there were no significant differences (P=0.05) of either *Fusarium* or *Penicillium* seedcoat colonization among the three seed size categories (table 2). Bleach treatment significantly reduced *Fusarium* seed colonization on both seedlots. However, *Penicillium* colonization was only significantly reduced in seedlot 7293. Stratification generally resulted in increased levels of both *Fusarium* and *Penicillium* on seeds (table 2); apparently these fungi were capable of spreading during stratification, resulting in high infection levels.

Standard nursery germination tests indicated that both seedlots severely infected with *Fusarium* had low germination (tables 3 and 4) compared to usual rates at the Coeur d'Alene Nursery. Germination on agar media used to isolate associated organisms were higher, but still relatively less than would be expected from healthy seedlots.

Seed size only affected germination for seedlot 7295 (table 4); small seed from this seedlot germinated at lower levels than either medium or large seed (tables 3 and 4). Although germination differences between medium and large seed were not significantly different, the trend was that larger seed germinated at higher rates. Previous studies (Dumroese and Wenny 1987; Griffin 1972) indicated that large pine seeds germinated more frequently than either medium or small seeds. However, Fowells (1953) found that medium-sized ponderosa pine seeds germinated best, whereas Larson (1963) found that, although large seeds yielded the largest seedlings, all seed sizes had nearly the same germination capacity and energy.

Cold-moist stratification is usually required to enhance germination of conifer seed in nurseries (Fuller and Hildebrand 1985; Kliejunas 1985). This procedure mimics natural conditions that occur in forests when seeds are disseminated in the fall and germinate the following spring (Wang 1988). Different conifer species have different stratification requirements to break dormancy and allow germination. In most germination tests of the current study, stratified seed germinated at the same or higher levels than unstratified seed (tables 3 and 4). Although stratified seed were colonized with high levels of *F. proliferatum* (table 2), this fungus apparently did not consistently affect germination.

Isolating high levels of *F. proliferatum* from conifer seeds was unusual (James and others 1995). Although this important species is commonly associated with seedling diseases, especially of container-grown stock in greenhouses (James 1997; James and others 1995, 1997), it is not normally an important seed contaminant (James and others 1991, 1995). *Fusarium proliferatum* produces abundant microconidia in chains and false heads (Elmer 1995; Nelson and others 1983). Spores become dry as mycelia age and are readily disseminated in air currents (Elmer and Ferrandino 1992; Hsieh and others 1979). Spores can dislodge from fungal hyphae occurring on plant material and easily spread. Experience in conifer seedling greenhouses

indicates that presence of this fungus increases throughout the seedling growth cycle so that by the time seedlings are removed from containers, many of them have roots extensively infected with *F. proliferatum* (Dumroese and others 1993; James 1997; James and others 1991). It is possible that relatively low levels of this fungus occurred on a few ponderosa pine seed and spread within specific lots, especially 7293 and 7295, during seed processing and stratification.

Table 1. Occurrence of *Fusarium* spp. on selected ponderosa pine seedlots from the USDA Forest Service Nursery, Coeur d'Alene, Idaho.

Seedlot	Percent Seeds Infected	Number Seeds Sampled
7293	34	196
7295	28	178
7305	1	100
7319	2	200
7321	1	200
7325	3	200
7338	2	152
7341	0	200
All Seedlots	9.5	1426

Table 2. Effects of seed size, bleach treatment and stratification on occurrence of *Fusarium* and *Penicillium* spp. on seedcoats of ponderosa pine seedlots 7293 and 7295 from the USDA Forest Service Nursery, Coeur d'Alene, Idaho[1].

Seedlot 7293	*Fusarium*[2]	*Penicillium*[2]	No Fungi[3]
Small Seed	41 A	42 A	-
Medium Seed	36 A	46 A	-
Large Seed	38 A	49 A	-
Bleach Treatment	3 A	34 A	63
Rinse Treatment	74 B	57 B	0
Stratified[4]	90 A	69 A	-
Unstratified[4]	58 B	45 B	-
Seedlot 7295			
Small Seed	23 A	49 A	-
Medium Seed	27 A	62 A	-
Large Seed	31 A	55 A	-
Bleach Treatment	2 A	67 A	63
Rinse Treatment	52 B	76 A	0
Stratified[4]	55 A	92 A	-

Unstratified[4]	48 A	61 B	-

[1]Figures in table are average percentage of sampled seed colonized with appropriate fungi.

[2] Within each column for each seed size and treatment group, means followed by the same capital letter are not significantly different (P=0.05) using a one-way analysis of variance and Duncan's multiple-range comparison test. Analyses were conducted separately for each seedlot.

[3]Percent of sampled seed with no fungi isolated from seedcoats (applicable only for bleach vs. rinse treatments since all other treatments yielded seedcoat fungi)

[4]Percent of stratified and unstratified water-rinsed seed only.

Table 3. Effects of seed size, bleach treatment and stratification on germination of ponderosa pine seedlot 7293 from the USDA Forest Service Nursery, Coeur d'Alene, Idaho.

Seed Size	Treatment[1]	Stratification[2]	21-Day Germ. [3]	10-Day Germ. [4]
Small	Rinsed	Unstratified	44	26
Small	Rinsed	Stratified	38	84
Medium	Rinsed	Unstratified	38	41
Medium	Rinsed	Stratified	48	67
Large	Rinsed	Unstratified	45	55
Large	Rinsed	Stratified	41	68
Small	Bleach	Unstratified	45	42
Small	Bleach	Stratified	24	68
Medium	Bleach	Unstratified	53	50
Medium	Bleach	Stratified	33	68
Large	Bleach	Unstratified	36	55
Large	Bleach	Stratified	55	65

Summaries of 21-day nursery germination (overall average germination=41.5%)[5]:

Seed size:	Average	Treatment[1]	Average	Stratification[2]	Average
Small	38 A	Bleach	41A	Stratified	40 A
Medium	43 A	Rinsed	42 A	Unstratified	43 A
Large	44 A				

Summaries of 10-day nursery germination (overall average germination=41.5%)[5]:

Seed size:	Average	Treatment[1]	Average	Stratification[2]	Average
Small	55 A	bleach	58 A	Stratified	70 A
Large	57 A	Rinsed	57 A	Unstratified	45 B
Medium	61 A				

[1]Rinsed = soaked in running water rinse for 48 hrs. following stratification and prior to germination tests; Bleach = treated with a solution of aqueous sodium hypochlorite (one part commer- cial bleach, two parts water) following stratification and prior to germination tests.

[2]Stratified = subjected to cool, moist stratification for 28 days using standard nursery procedures.

[3]Standard nursery germination tests conducted on moisted cotton in plastic dishes within an environmentally-controlled growth chamber.

[4]Germination evaluated from seeds incubated on Komada's medium under diurnal cycles of fluorescent light at about 24°C for 10 days.

[5]Within each seed size and treatment group, means followed by the same capital letter are not significantly different (P=0.05) using a one-way analysis of variance and Duncan's multiple-range comparison test. Analyses were conducted separately for 21- and 10-day germination tests.

Table 4. Effects of seed size, bleach treatment and stratification on germination of ponderosa pine seedlot 7295 from the USDA Forest Service Nursery, Coeur d'Alene, Idaho.

Seed Size	Treatment[1]	Stratification[2]	21-Day Germ.[3]	10-Day Germ.[4]
Small	Rinsed	Unstratified	14	28
Small	Rinsed	Stratified	12	40
Medium	Rinsed	Unstratified	40	32
Medium	Rinsed	Stratified	9	42
Large	Rinsed	Unstratified	32	21
Large	Rinsed	Stratified	24	52
Small	Bleach	Unstratified	14	7
Small	Bleach	Stratified	4	30
Medium	Bleach	Unstratified	21	31
Medium	Bleach	Stratified	16	45
Large	Bleach	Unstratified	37	23
Large	Bleach	Stratified	15	56

Summaries of 21-day nursery germination (overall average germination = 19.8%)[5]:

Seed size:	Average	Treatment[1]	Average	Stratification[2]	Average
Small	11 A	Bleach	18 A	Stratified	13 A
Medium	21 B	Rinsed	22 A	Unstratified	26 B
Large	27 B				

Summaries of 10-day agar germination (overall average germination = 33.9%)[5]:

Seed size	Average	Treatment[1]	Average	Stratification[2]	Average
Small	26 A	Bleach	32 A	Stratified	44 A
Medium	38 B	Rinsed	36 A	Unstratified	24 B
Large	38 B				

[1]Rinsed = soaked in running water rinse for 48 hrs. following stratification and prior to germination tests; Bleach = treated with a solution of aqueous sodium hypochlorite (one part commercial bleach, two parts water) following stratification and prior to germination tests.

[2]Stratified = subjected to cool, moist stratification for 28 days using standard nursery procedures.

[3]Standard nursery germination tests conducted on moisted cotton in plastic dishes within an environmentally-controlled growth chamber.

[4]Germination evaluated from seeds incubated on Komada's medium under diurnal cycles of fluorescent light at about 24^{o}C for 10 days.

[5]Within each seed size and treatment group, means followed by the same capital letter are not significantly different (P=0.05) using a one-way analysis of variance and Duncan's multiple-range comparison test. Analyses were conducted separately for 21- and 10-day germination tests.

Like other fusaria associated with conifer seedling diseases, not all *F. proliferatum* isolates are aggressive pathogens, although when environmental conditions are conducive for fungal development, most isolates may exhibit high virulence on susceptible conifer germinants (James 1997; James and others 1997). It is possible that some contaminated seed were internally infected with *F. proliferatum*. If such were the case, seed likely became initially infected during formation within cones (Anderson and others 1980; 1984; Fraedrich and Miller 1995; Sutherland 1991). Even a small percentage of seed infection might result in large levels of contamination with a fungus that spreads as readily as *F. proliferatum*.

To reduce potential damage resulting from *Fusarium*-infected seed, it is important that growers evaluate potential for these pathogenic fungi on seed germination and seedling establishment. Seedlots that germinate at less than expected levels should be evaluated for presence and extent of *Fusarium* contamination. If lots have more than 10% of their randomly selected seed contaminated with *Fusarium,* they should be treated to reduce contamination. Treatments should be applied prior to stratification to reduce potential for fungal spread. Although running-water rinses and bleach treatments may reduce contamination, sufficient inoculum may survive treatment to cause disease problems after sowing (Campbell and Landis 1990; James and Genz 1981). Hydrogen peroxide has also been used to surface sterilize conifer seed (Ching and Parker 1958; Edwards and Sutherland 1979; Fuller and Hildebrand 1985), although in some cases phytotoxicity to young germinants outweighs advantages of reducing surface fungal populations (Campbell and Landis 1990). Several fungicides have also been used to treat fungus-contaminated conifer seed (Bloomberg and Trelawny 1970; Cooley 1980; Cram and Vaartaja 1955; Hamilton and Jackson 1951). However, problems with adverse effects on germination (Pawuk 1979; Peterson 1970) and phytotoxicity have resulted in most growers avoiding use of fungicides on seeds (Campbell and Landis 1990).

Seed treatments with potential biocontrol agents show promise in reducing seed-borne diseases.

Selected biocontrol agents must be antagonistic toward pathogens normally occurring on seeds (Taylor and Harman 1990) and not adversely affect seed performance (Harman 1991; Taylor and Harman 1990). Biocontrol agents showing promise on seeds of agricultural crops include selected bacteria (Pokorny and Rykhus 1993; Taylor and Harman 1990) and fungi (Harman 1991; Sutherland and van Eerden 1980). Unfortunately, some of these have not performed as well in controlling conifer seedling diseases as they have in other plant pathosystems (Dumroese and others 1996, 1998). To reduce losses from pathogenic organisms, additional work is needed to identify more effective biocontrol agents for conifer seedling nurseries, as well as to develop improved delivery systems.

Seed-processing equipment (cone storage and drying, seed extraction and purifying, seed storage) may be contaminated with potentially pathogenic fungi. If so, successive seedlots being processed may become contaminated during processing. This may be especially important with fungal species, such as *F. proliferatum*, that profusely produce spores that are easily disseminated. A relatively small amount of initial inoculum can result in high levels of seed contamination. Therefore, periodic cleaning of seed-processing equipment is important to reduce threat of fungal spread. Seed extraction tumblers should be cleaned periodically to remove as much organic matter as possible. Reduced periods of cone storage are important (Harvey and Carpenter 1975; James 1995; Miller and Bramlett 1975; Peterson and Pigott 1996; Rediske and Shea 1965). Since potentially pathogenic fungi may spread during cold seed storage and stratification (Campbell and Landis 1990; Kliejunas 1985, 1987), reducing storage and stratification time may reduce potential damage from seed-colonizing fungi (James 1983, 1995; Miller and others 1984). Experience has shown that santitaion is an important disease-prevention tool in nurseries (James and others 1990a, 1991). Keeping equipment and growing environments clean and relatively pathogen-free will go a long way in reducing impacts of pathogenic fungi on seedling production.

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